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CCL9/MIP-1γ and Its Receptor CCR1 are the Major Chemokine Ligand/Receptor Species Expressed by Osteoclasts

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Abstract Although much has been learned recently of the mechanisms by which the differentiation of osteoclasts is induced, less is known of the factors that regulate their migration and localization, and their interactions with other bone cells. In related cell types, chemokines play a major role in these processes. We therefore systematically tested the expression of RNA for chemokines and their receptors by osteoclasts. Because bone is the natural substrate for osteoclasts and may influence osteoclast behavior, we also tested expression on bone slices. Quantitative RT-PCR using real-time analysis with SYBR Green was therefore performed on RNA isolated from bone marrow cells after incubation with macrophage-colony stimulating factor (M-CSF) with/without receptor-activator of NFκB ligand (RANKL), on plastic or bone. We found that RANKL induced expression of CCL9/MIP-1γ to levels comparable to that of tartrate-resistant acid phosphatase (TRAP), a major specialized product of osteoclasts. CCL22/MDC, CXCL13/BLC/BCA-1, and CCL25/TECK were also induced. The dominant chemokine receptor expressed by osteoclasts was CCR1, followed by CCR3 and CX3CR1. Several receptors expressed on macrophages and associated with inflammatory responses, including CCR2 and CCR5, were down-regulated by RANKL. CCL9, which acts through CCR1, stimulated cytoplasmic motility and polarization in osteoclasts, identical to that previously observed in response to CCL3/MIP-1α, which also acts through CCR1 and is chemotactic for osteoclasts. These results identify CCL9 and its receptor CCR1 as the major chemokine and receptor species expressed by osteoclasts, and suggest a crucial role for CCL9 in the regulation of bone resorption. J. Cell. Biochem. 87: 386–393, 2002.

Key words: monocytes/macrophages; chemokines; cellular activation; cellular differentiation

The structural integrity of the skeleton is maintained by the co-ordinated activity, during bone remodeling, of osteoclasts which remove bone, and osteoblasts which replace it. Much has been learned recently of the osteoblast-derived signals that induce osteoclastic differentiation. It is now known that osteoclast formation occurs when bone marrow cells are incubated with macrophage-colony stimulating factor (M-CSF) and receptor-activator of NF-κB ligand (RANKL), a novel member of the TNF-superfamily that is expressed by osteoblastic cells [Suda et al., 1999; see Chambers, 2000 for reviews]. However, much less is known of the mechanisms governing such processes as osteoclastic recruitment and localization, and co-ordination of bone resorption, or of the signals through which osteoclasts communicate with osteoblastic cells.

In other tissues, under both inflammatory and homeostatic circumstances, chemokines play a major role in such processes. Chemokines are a superfamily of small, cytokine-like proteins that induce, through their interaction with G-protein-coupled receptors, cytoskeletal rearrangement, adhesion, and directional migration in leukocytes and other cells, including cells closely related to osteoclasts, such as monocytes and macrophages [Butcher et al., 1999; Campbell and Butcher, 2000; Rossi and Zlotnik, 2000; Zlotnik and Yoshie, 2000]. It therefore seems likely that they will be found to play a similar role in the co-ordination of the activity of bone cells. Failure of this co-ordination, either intrinsic or when perturbed by inflammation, may disrupt bone remodeling and lead to the

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bone loss and structural failure seen in diseases such as postmenopausal osteoporosis, inflammatory osteolysis, and metastatic bone disease.

Despite their potential importance in such diseases, little is known of the chemokine expression and responsiveness of osteoclasts. This is largely due to the difficulty, until recently, of obtaining osteoclasts in sufficient purity and numbers for analysis. However, with identification of RANKL, the osteoclast-inductive cytokine, it has become possible to generate osteoclasts in large numbers and uncontaminated by stromal and other unrelated cells. We therefore exploited the opportunity provided by the identification of RANKL, to test the expression by osteoclasts of RNA for chemokines and their receptors.

It is known that the behavior of cells is substantially influenced by the nature of the substrate on which they are incubated. Although osteoclasts can be induced to differentiate from bone marrow cells on plastic substrates, their natural substrate is bone, and it is clear that osteoclastic differentiation differs at least quantitatively on bone, versus plastic, and might well also differ qualitatively [Fuller and Chambers, 1989; Hentunen et al., 1994; Fuller et al., 2000]. Moreover, the osteoclast might express distinct patterns of chemokines during bone resorption for important functions such as the feedback regulation of bone resorption, or the induction of bone formation. The latter is part of the coupling between resorption and formation, that is essential to bone remodeling.

We therefore assessed expression of chemokines and receptors by osteoclasts incubated on bone slices. Because the relatively small surface area of bone slices imposes practical limits on the amount of RNA that can be extracted, RNA expression was analyzed by real-time PCR. This also has the advantage, especially compared to comparative approaches such as gene arrays, in providing quantitative data on the RNA species expressed. We found that chemokine ligands CCL9 (MIP-1γ), CCL12 (MCP-5), and CCL25 (TECK), and chemokine receptors CCR1, CCR3, and CX3CR1 were the species most highly expressed by osteoclasts on bone. Expression of CCL9, CCR1, and CCR3 were strongly induced, while CCR2, CCR5, and CCR7 were inhibited, by RANKL. The induction of expression of substantial levels of CCL9 and its receptors in osteoclasts suggests an important role for this chemokine in bone physiology or pathology.

MATERIALS AND METHODS

Media and Reagents

Non-adherent, M-CSF-dependent bone marrow cells were incubated with MEM with Earle's salts (EMEM) (Sigma, Poole, Dorset, UK) supplemented with 10% FCS (Autogen Bioclear, Calne, Wiltshire, UK). HEPES-buffered medium 199 (Sigma) was used for isolation and sedimentation of osteoclasts ex vivo, and EMEM for subsequent time-lapse observations. All media were supplemented with 2 mM glutamine, 100 IU/ml benzylpenicillin, and 100 μg/ml streptomycin (Sigma). Incubations were performed at 37°C in 5% CO_2 in humidified air. Recombinant human M-CSF was provided by Chiron Corp. (Emeryville, CA); soluble recombinant murine RANKL was from Amgen Inc (Thousand Oaks, CA). Recombinant murine CCL9/MIP-1γ was from R&D Systems (Abingdon, Oxon, UK). Slices of bovine cortical bone were prepared as previously described [Chambers et al., 1985].

Isolation and Culture of Bone Marrow Cells and Preparation of RNA

Bone marrow cells were isolated from male MF1 mice and cultured as previously described [Wani et al., 1999]. Briefly, male MF1 mice (5–8-week-old) were killed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of soft tissue. The bone ends were cut, and the marrow cavity was flushed out into a Petri dish by slowly injecting PBS at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated through a 21-gauge needle to obtain a single cell suspension. Bone marrow cells were then washed, resuspended in EMEM/FCS, and incubated at a density of 3×10^5 cells/ml for 24 h in a 75 cm^2 flask (Helena Biosciences, Sunderland, Tyne & Wear, UK) with M-CSF (5 ng/ml). After 24 h, non-adherent cells were harvested, washed, and resuspended in EMEM/FCS. The cells were placed in the wells of a 24-well plate (Helena Biosciences) (2×10^5 cells per well) and incubated in a total volume of 1 ml in M-CSF (30 ng/ml) with/without RANKL (50 ng/ml) either on the well base or on bone slices (1 cm × 1 cm) placed in the well. Cultures were fed every 2–3 days by replacing 600 μl of culture medium with an equal volume of fresh medium and cytokines. After 5 days, osteoclastic differentiation was

confirmed in sample cultures by TRAP histochemistry or bone resorption as described previously [Wani et al., 1999]. The remaining cultures were washed to remove non-adherent cells, and used to harvest RNA. RNA was harvested using RNAeasy Minikits (Qiagen, Crawley, UK), following manufacturer's instructions and treated with DNase 1 (Life Technologies). Each batch of RNA was prepared by pooling extracts from 24 wells. Total RNA was analyzed for purity and quantified spectrophotometrically.

Assessment of RNA Expression

Primers were designed using primer design programs (CP Primers (for Apple Macintosh) or Primer 3 (for PC)), using murine sequences obtained from Genbank database (see Table I). For assessment of RNA expression, 2 μg of total RNA was reverse transcribed for 1 h at 42°C using 100 pmoles random hexamers (Amersham Biosciences, Bucks, UK), 600 U MMLV (Life Technologies, Paisley, UK), in a 50 μl reaction. This was then diluted to a final volume of 100 μl.

TABLE I. Chemokine/Chemokine Receptor Primer Sequences, Melting Temperatures Used, Predicted Product Lengths, and Accession Numbers

	Tm	Product length	Forward primer	Reverse primer	Genbank accession no.
Chemokines					
CCL1 (I-309)	64	132	ccgtgtggatacaggatgttg	tcaggacaggaggagccc	NM_011329
CCL2 (MCP-1)	60	247	ttttgtcaccaagctcaagagag	tcactgtcacactggtcactcc	NM_011333
CCL3 (MIP 1α)	59	201	accactgcccttgctgttc	tctgccggtttctcttagtcag	NM_011337
CCL4 (MIP 1β)	59	229	ctctcctcttgctcgtggc	gtactcagtgacccagggctc	M35590
CCL5 (RANTES)	60	236	gctgccctcaccatcatcc	gtattcttgaacccacttcttctctg	M77747
CCL6 (MRP-1)	59	225	gtggctgtccttgggtcc	agacctgggttcccctcc	NM_009139
CCL7 (MCP-3)	59	256	gctcatagccgctgctttc	gctttggagttggggttttc	Z12297
CCL8 (MCP-2)	59	145	ccagataaggctccagtcacc	agagagacatacctgcttggtc	NM_021443.1
CCL9 (MIP 1γ)	59	193	caacagagacaaaagaagtccagag	cttgctgataaagatgatgccc	NM_011338
CCL11 (Eotaxin)	59	190	gtcacttccttcacctcccag	atctctttgcccaacctggt	NM_011330
CCL12 (MCP-5)	54	148	atgcctcctgctcatagc	ggctgcttgtgattctcc	NM_011331
CCL17 (TARC)	64	210	ctgctctgcttctggggac	tgtttgtctttggggtctgc	AJ242587
CCL19 (MIP 3β)	66	182	gctggttctctggaccttcc	gctgatagcccctagtgtgg	AF307988
CCL20 (MIP 3α)	65	198	gcagccaggcagaagcagc	tcacagccctttcacccagttc	NM_016960
CCL21 (6CKINE)	63	133	gcagtgatggaggggtcag	cggggtgagaacaggattgc	AF006637
CCL22 (MDC)	59	132	ggtccctatggtgccaatg	ttatcaaaacaacgccaggc	NM_009137
CCL25 (TECK)	59	245	tgaaactgtggctttttgcc	gtcaagattctcatcgccctc	NM_009138
CCL27 (CTACK)	63	288	gccttgcctctgccctcc	gttttgctgttgggggtttgag	NM_011336
CX3CL1 (FRAKTALKINE)	59	290	cctcactaaaaatggtggcaag	atgtcagccgcctcaaaac	MMU92565
XCL1 (LYMPHOTACTIN)	65	293	gacttctcctcctgactttcctcctggg	tgctggtggacctctggc	NM_008510
CXCL1 (GROα)	63	196	cacccgctcgcttctctg	cttgagtgtggctatgacttcgg	NM_008176
CXCL2 (GRO beta) (MIP 2α)	59	159	caccaaccaccaggctacag	gcccttgagagtggctatgac	NM_009140
CXCL4 (PF4)	59	111	gtgtgaagaccatctcctctgg	cattcttcagggtggctatgag	NM_019932
CXCL6 (GCP-2)	59	167	ttctgttgctgttcacgctg	ccaccgtagggcactgtg	NM_009141
CXCL9 (MIG)	59	320	cttcctggagcagtgtggag	cgactttggggtgttttgg	NM_008599
CXCL10 (CRG-2)(IP-10)	59	152	gactcaagggatccctctcg	cctgctgggtctgagtggg	NM_021274
CXCL11 (I-TAC)	59	193	ggtcacagccatagccctg	agccttcatagtaacaatcacttcaac	NM_019494
CXCL12 (SDF-1)	59	167	cacatcgccagagccaac	catcttgagcctcttgtttaaagc	NM_013655
CXCL13 (BCA-1)	59	252	aggctcagcacagcaacg	tctttgtaaccatttggcacg	NM_018866
CXCL14 (BRAK) (BOLOKINE)	59	101	actgcgaggagaagatggttatc	tgaagcgtttggtgctctg	AF152377
CXCL15 (LUNGKINE)	56	359	acgatgtctgtgtattcaggaac	tgtgagctaaatcagcaaagtg	NM_011339
Chemokine receptors					
CCR1	59	390	aagagcctgaagcagtggaag	gcagccattttgccagtg	NM_009912
CCR2	59	265	ccacaccctgtttcgctg	accttcggaacttctctccaac	NM_009915
CCR3	59	245	tcctctcctcgttatggttatctg	gtgtaggcaatcacctcatcagtcac	NM_009914
CCR4	59	123	gcctcttgttcagcacttgc	ataagcagccccaggacg	NM_009916
CCR5	59	206	ctgccaaaaaatcaatgtgaaac	tgagcccagaatggtagtgtg	NM_009917
CCR6	59	253	ttggaacggatgattatgacaac	cggtagggtgaggacaaagag	NM_009835
CCR7	59	206	ctacgaaagcatgccaaagc	aggacgaacagcaaatccg	NM_007719
CCR8	59	162	acagcctggtcatcttagtcctc	ttacacatcgcagtcccaaac	NM_007720
CCR10	59	251	cccagtgtctccctgatgg	gaagccagcgtggaaagag	NM_007721
CXCR2	59	251	cctggaaatcaacagttatgctg	tccttcacgtatgagaatatcttgc	L13239
CXCR3	59	219	cagcctgaactttgacagaacc	gccgaaaacccactggac	NM_009910
CXCR4	59	219	ctttgtcatcacactcccctt	gcccacatagactgccttttc	NM_009911
CX3CR-1	59	262	ttcattggcttctttgggg	atgttgacttccgagttgcg	AF074912
LYMPHOTACTIN RECEPTOR	59	264	tatccataccctccgctgc	atgagcctgactgttcggtg	NM_011798
Other					
TRAP	58	218	tcccctggtatgtgctgg	gcattttgggctgctgac	NM_007388
c-fms	59	188	tgctaaagtccacggctcat	tcggagaaagttgagatggtgt	NM_007779
CD-16	63	149	gcgggtgtttctggaaggg	ctgtggttggcttttgggatagag	M14215
β-actin	60	197	gtcatcactattggcaacgag	cctgtcagcaatgcctgggtacat	M12481
GAPDH	60	217	cggatttggccgtattgg	ggtctcgctcctggaagatg	NM_008084

Real-time PCR was carried out using the I-Cycler (BioRad, Hemel Hempstead, Herts, UK) using the DNA binding dye SYBR Green for the detection of PCR products. Two microliters of either external plasmid standards or a cDNA (equating to 40 ng of total RNA) was added to a final reaction volume of 25 μl containing 200 μM dNTPS, 200 μM primers, 0.25 U AmpErase UNG and 2.5 μl 10 × SYBR Green PCR buffer, 3 mM $MgCl_2$, and 0.625 U of Platinum Taq polymerase (Universal PCR Master mix; Applied Biosystems, Warrington, UK). This was then made up to 100 μl, and 2 μl of this was used as a template for 25 μl PCR reactions.

For the generation of standard curves, plasmid clones containing the corresponding chemokine cDNA were constructed. Briefly, total RNA was extracted from known positive tissues (mostly thymus and bone marrow) using the PCR primers described in Table I. The amplicons were then cloned into PGEM Teasy (Promega, Southampton, UK). The concentration of DNA plasmid stock was determined by the optical density at 260 nm. Copy number for each plasmid was calculated on the spectrophotometric reading. The linear range of the assay was determined by the amplification of log serial dilutions of plasmids from 500 to 5×10^6. The progress of the PCR amplification was monitored by real-time fluorescence emitted from the SYBR Green during the extension time. Typically, the cycles were 95° for 3 min, followed by 40 cycles of 95° for 20 s, 59° for 20 s, and 72° for 20 s.

For each sample, mRNA levels of each chemokine or receptor were expressed as relative copy number normalized against GAPDH mRNA. This was achieved by constructing a standard curve for each PCR run from serial dilutions of purified plasmid DNA with specified amplicon. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. Samples were analyzed in triplicate. For each sample, chemokine copy number relative to the GAPDH copy number in the same sample was calculated.

At the end of each PCR run, a melt curve analysis was performed. Gel electrophoresis was also performed to confirm the correct size of the amplicon and the absence of non-specific bands.

To confirm the PCR results, Northern analysis was performed on species of interest. For this, total RNA was extracted from cultures incubated on plastic using Trizol (Life Technologies) according to the manufacturer's instructions. Twenty micrograms of total RNA was blotted and hybridized as previously described [Lean et al., 2000]. The probes used were the amplicons isolated after subcloning from the vector. Probes were labeled by Megaprime DNA labeling system (Amersham) with [α-^{32}P]dATP (Amersham).

Isolation and Observation of Osteoclasts Ex Vivo

Osteoclasts were disaggregated from the long bones of 2–3-day-old Wistar rats, as previously described [Chambers et al., 1985]. For this, the femora, tibiae, and humeri were removed and dissected free of adherent tissue. Bones were then cut across their epiphyses and curetted into medium 199. The curettings were agitated with a Pasteur pipette. Large fragments were allowed to sediment for 10 s. The cell suspension was then transferred to a 25 cm^2 tissue culture flask (Helena Biosciences) and incubated for 15 min. Non-adherent cells were removed by washing with PBS. Five milliliters of EMEM/FCS were placed in the flask and the cells were incubated for 30 min. Flasks were then sealed and placed in the incubation chamber of an Olympus IMT-373 inverted microscope. A suitable osteoclast-containing field was chosen and recorded for 45 min on a time-lapse video recorder. Vehicle or chemokine was added in 0.5 ml of prewarmed EMEM/FCS and recording continued.

RESULTS

Although osteoclasts form when bone marrow precursors are incubated on plastic in M-CSF with RANKL, a significantly higher proportion of precursors become osteoclastic if incubated on bone slices [Fuller et al., 2000]. Therefore, we initially assessed expression by cultures of bone marrow cells incubated on bone slices in the presence of RANKL and M-CSF. For each RNA preparation, the presence of large numbers of osteoclasts was confirmed in sample bone slices by staining for TRAP, and inspecting the bone surface for excavations. Only those RNA samples from experiments that showed substantial (>10% of the bone surface) bone resorption were used.

We found that CCL9 was the chemokine most highly expressed, followed by CCL25 and CCL12 (Fig. 1). Chemokine receptors CCR1,

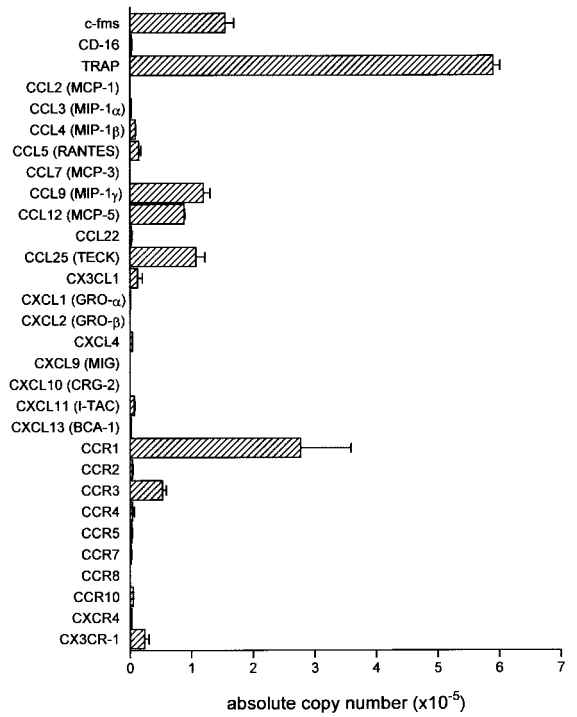

Fig. 1. Expression of chemokine/chemokine receptors by murine bone marrow cells after incubation on bone slices for 5 days in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml). RNA extracted from 24 cultures was pooled, reverse transcribed, and analyzed by real-time quantitative PCR. Values are expressed as absolute copy number per 20 ng of total RNA.

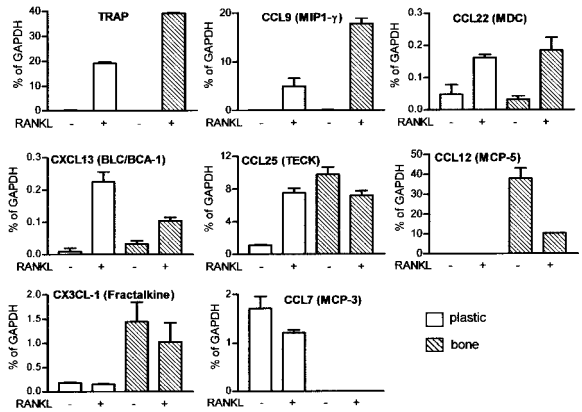

Fig. 2. Expression of RNA for chemokine ligands by bone marrow cells after incubation on plastic (open histograms) or bone slices (hatched histograms) for 5 days in M-CSF (30 ng/ml) with/without RANKL (50 ng/ml). Results are expressed as percent of expression of RNA for GAPDH (mean ± SEM) of 24 pooled cultures per variable. Each result was confirmed using at least two further batches of RNA.

CCR3, and CX3CR1 were most strongly expressed, at a level an order of magnitude greater than expression of the remaining receptors, including CCR2, CCR4, and CCR5. CCR1 was expressed at a similar level to c-fms, suggesting that this is the major chemokine receptor in osteoclasts.

Osteoclastic differentiation is increased quantitatively by incubation on bone [Fuller et al., 2000]. It may also be changed qualitatively. Therefore, to determine the contribution of bone versus RANKL to chemokine/chemokine receptor expression, we compared their expression in cultures of bone marrow cells incubated in M-CSF, with versus without RANKL, on plastic and on bone. To facilitate comparison between groups, results were expressed as a ratio to GAPDH in the same RNA preparation. We found a striking induction of expression of CCL9 by RANKL (Fig. 2). This was augmented by incubation on bone. This pattern is very similar to that of TRAP, suggesting that CCL9 expression is a 'professional' activity of osteoclasts. Expression of CCL22 and CXCL13 was also increased by RANKL, but at levels two orders of magnitude lower than CCL9. CCL25 appeared to be inducible by either RANKL or bone. Expression of CCL12 and CX3CL1 seemed to be induced by incubation of bone marrow cells on bone, while CCL7 showed the reverse pattern (Fig. 2). Expression of the remaining chemokines and receptors tested was either undetectable, or unaffected by culture conditions.

Two chemokine receptors, CCR1 and CCR3, showed clear and reproducible induction by RANKL, especially in cells incubated on bone (Fig. 3). In contrast, expression of several receptors (CCR2, CCR5, and CCR7) was, like the macrophage receptor CD16, strongly inhibited by RANKL. CCR10, CXCR4, and CX3CR1 appeared to be induced by incubation of bone marrow cells on bone; expression on bone was reduced by RANKL. The remaining receptors tested were unaffected by culture conditions.

Expression of CCL9, CCR1, and CCR3, which were all strongly induced by RANKL as judged by real-time PCR, was also assessed by Northern analysis. Northern analysis confirmed strong induction of RNA for these species by RANKL (Fig. 4).

The expression of CCL9 and its receptor (CCR1) suggests an autocrine or paracrine role for this chemokine in osteoclasts. We found that addition of CCL9 to osteoclasts caused a rapid and dramatic change in their behavior, resembling that seen in other cell types in response to chemotactic agents [Zigmond and Sullivan, 1979; Shields and Halston, 1985] (Fig. 5).

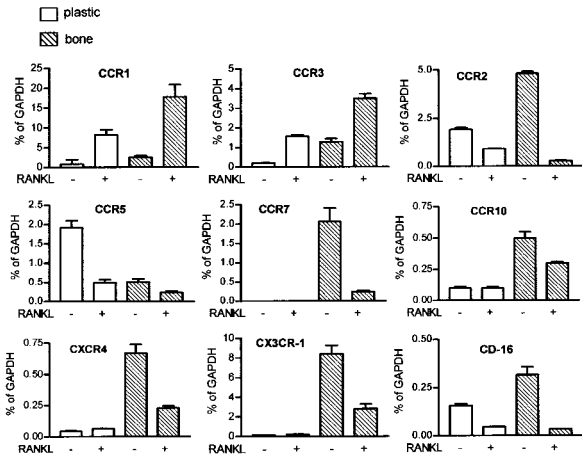

Fig. 3. Expression of RNA for chemokine receptors by bone marrow cells after incubation on plastic (open histograms) or bone slices (hatched histograms) for 5 days in M-CSF (30 ng/ml) with/without RANKL (50 ng/ml). Results are expressed as percent of expression of RNA for GAPDH (mean ± SEM) of 24 pooled cultures per variable. Each result was confirmed using at least two further batches of RNA.

Within minutes, osteoclasts incubated in CCL9 (100 ng/ml) showed a dramatic increase in cytoplasmic motility, associated with increased cell spreading. Cells then developed a polarized appearance, and migrated in the direction of the broad, leading pseudopods (Fig. 5). This response was observed in all six preparations observed. Similar to our previous experience testing CCL3 (MIP-1α) [Fuller et al., 1995],

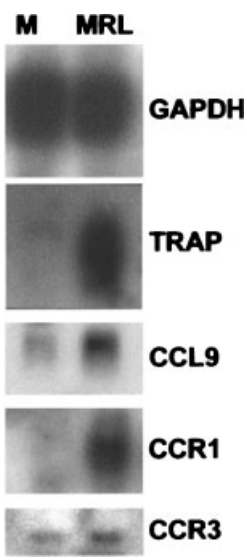

Fig. 4. Northern analysis of GAPDH, TRAP, CCL9, CCR1, and CCR3 expression by bone marrow cells incubated for 5 days in tissue culture flasks in M-CSF (30 ng/ml) with (MRL)/without RANKL (50 ng/ml).

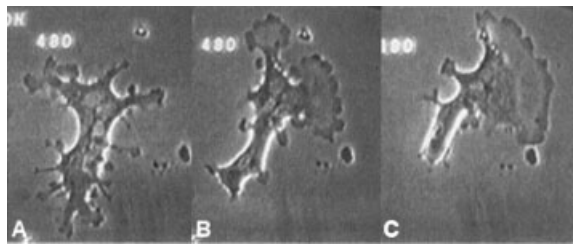

Fig. 5. Effect of CCL9 on osteoclastic behavior. Osteoclasts were extracted from newborn rat long bones and recorded by time-lapse video phase-contrast light microscopy. Figure shows an osteoclast immediately before (**A**) and 30 (**B**), or 60 min (**C**) after addition of CCL9 (100 ng/ml). After addition of CCL9, the non-polarized osteoclast developed a polarized morphology (B,C), and migrated in the direction of the pole that had developed expanded pseudopods.

CCL9 did not induce osteoclast formation from bone marrow cells in the presence of M-CSF alone; and did not change either the number of TRAP-positive multinuclear cells formed or bone resorption by bone marrow cells incubated on plastic or bone slices, in the presence of M-CSF and RANKL (data not shown).

DISCUSSION

Bone depends on the continuous and coordinated activities of bone-resorbing osteoclasts and bone-forming osteoblasts. Chemokines have been found to play a crucial role in the coordination of the localization and activity of cells in many tissues, but very little is known about the chemokines and receptors expressed by osteoclasts. A major impediment has been lack of availability of osteoclasts in sufficient number or purity for the analysis. We have therefore exploited the opportunity provided by the identification of RANKL, the osteoclast-inductive ligand, to systematically assess the expression of chemokines and their receptors in osteoclasts.

We identified CCL9 as the major chemokine expressed by osteoclasts. CCL9, which was virtually undetectable without RANKL, was expressed by osteoclasts at levels comparable to those of TRAP, a major secreted product of osteoclasts. Intriguingly, the chemokine receptor most strongly induced by RANKL was CCR1, the cognate receptor for CCL9 [Youn and Kwon, 2000], followed by CCR3. We also found that CCL9 activated cytoplasmic motility and spreading in osteoclasts isolated from rat bone, eliciting an identical response to that previously observed with M-CSF and CCL3 which are

chemoattractant for osteoclasts [Fuller et al., 1993, 1995]. Like CCL9, CCL3 can signal through CCR1. This suggests that CCL9 might play an autocrine–paracrine role in chemoattraction and accumulation of osteoclasts and their precursors at resorptive sites.

Recently, it was reported that CCL3, a factor produced by multiple myeloma cells, may play an important role in osteoclast formation and bone destruction in multiple myeloma [Choi et al., 2001]. There is also evidence that osteoblastic cells, which govern osteoclastic localization and activity, express CCL3 [Kukita et al., 1997; Scheven et al., 1999]. Thus, high expression of CCR1 by osteoclasts might enable an autocrine–paracrine amplification by osteoclastic CCL9 of an osteoblast-derived-CCL3 chemoattractant signal. Pathological mimicry of this physiologic bone regulatory mechanism by multiple myeloma cells might contribute to bone loss in multiple myeloma.

We also noted that while RANKL upregulates CCR1 and CCR3, it strongly downregulates expression of CCR2 and CCR5. Lack of expression of CCR2 by osteoclasts is consistent with the reported failure of the inflammatory chemokines CCL2, CCL7, and CCL8, which all signal through CCR2, to chemoattract osteoclast precursors [Votta et al., 2000]. Intriguingly, the same authors noted chemotactic responses, in osteoclast precursors, to CCL23 (which was reported to be expressed by bone tissue), CCL3, and CCL5. All these chemokines can signal through CCR1, which we have found to be highly expressed on osteoclasts. CCL3 and CCL5 can also signal through CCR5, which is highly expressed on macrophages but down-regulated by RANKL. This switching of chemokine receptors between macrophages and osteoclasts suggests that the response of the cells to the same chemokine may differ. We speculate that similar to the chemokine receptor switching noted in Th1 versus Th2 responses [Bonecchi et al., 1997; Sallusto et al., 1998; Zingoni et al., 1998], CCR5 might activate both chemoattraction and pro-inflammatory activities in macrophages, while chemoattraction without pro-inflammatory responses might be more appropriate in bone physiology, and might be mediated by CCR1. In fact, RANKL downregulates many receptors for pro-inflammatory chemokines, including not only CCR2 and CCR5 but also CCR7, CCR10, CXCR4, and CX3CR1. This suggests that, compared to macrophages, the osteoclast has a limited repertoire of responsiveness to inflammatory chemokines.

Induction by RANKL of CCR3 is surprising, since this is the principal receptor on eosinophils. Expression might represent an osteoclast characteristic. Alternatively, it might reflect the differentiation pattern of multinuclear giant cells associated with parasitic infections, which share some characteristics with osteoclasts, including strong cathepsin K expression [Diaz et al., 2000].

The osteoclast is surprisingly limited not only in expression of chemokine receptors, but also of chemokine ligands. Many of the chemokines were originally identified in inflammatory cells, and are pro-inflammatory. Lack of expression by osteoclasts is consistent with the lack of inflammatory cells in the resorptive microenvironment. Consistent with this, the dominant chemokine product CCL9 is constitutively expressed in vivo, in the absence of inflammation [Poltorak et al., 1995], and is thus unlikely to play a pro-inflammatory role. Several other chemokines were also induced by RANKL, including CCL22, CXCL13, and CCL25. The significance of the expression of these chemokine ligands by osteoclasts is unknown. However, while CCL22 was consistently upregulated by RANKL, expression was two orders of magnitude lower than CCL9. Nevertheless, low expression does not exclude a juxtacrine action.

Bone resorption and bone formation are tightly coupled processes, such that under normal circumstances bone resorption is followed, at the same site, by bone formation [Hattner et al., 1965; Parfitt 1982]. The function of this coupling is uncertain. It may serve to replace fatigue-damaged bone, or it may assist calcium homeostasis. Whatever its function, the skeleton depends for its integrity on an accurate balance between these processes. It is considered likely that the coupling of these processes occurs through the release of signals from osteoclasts during bone resorption, that prepare and activate osteoblastic cells to form bone. We did not find a chemokine whose expression corresponded to this pattern: chemokines produced by osteoclasts incubated on bone were also produced on plastic. This raises the possibility that there might be novel chemokines that mediate the coupling of bone resorption to bone formation. Whether or not this is so, we have identified CCL9 as the major species, amongst known chemokines, to be expressed, and at very

high levels, by osteoclasts. Moreover we have found that CCR1, the cognate receptor for this chemokine, is the major osteoclast-expressed chemokine receptor. These findings suggest that CCL9 plays an important role in the regulation of bone resorption.

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